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Reactions of Papain and of Low-Molecular-Weight Thiols with some Aromatic Disulphides

2,2′-DIPYRIDYL DISULPHIDE AS A CONVENIENT ACTIVE-SITE TITRANT FOR PAPAIN EVEN IN THE PRESENCE OF OTHER THIOLS

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1. The u.v.-spectral characteristics of 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2), 2,2′-dipyridyl disulphide (2-Py–S–S–2-Py), 4,4′-dipyridyl disulphide (4-Py–S–S–4-Py), 5-mercapto-2-nitrobenzoic acid (Nbs), 2-thiopyridone (Py–2-SH) and 4-thiopyridone (Py–4-SH) were determined over a wide range of pH and used to calculate their acid dissociation constants. 2. The reactions of L-cysteine, 2-mercaptoethanol and papain with the above-mentioned disulphides were investigated spectrophotometrically in the pH range 2.5–8.5. 3. Under the conditions of concentration used in this study the reactions of both low-molecular-weight thiols with all three disulphides resulted in the stoicheiometric release of the thiol or thione fragments Nbs, Py–2-SH and Py–4-SH at all pH values. The rates of these reactions are considerably faster at pH8 than at pH4, which suggests that the predominant reaction pathway in approximately neutral media is nucleophilic attack of the thiolate ion on the unprotonated disulphide. 4. The reaction of papain with Nbs_2 is markedly reversible in the acid region, and the pH-dependence of the equilibrium constant for this system in the pH range 5–8 at 25°C and $I = 0.1$ is described by:

$$K_{equil.} = 3.5 \times 10^{-3} (3.92 \times 10^{-5} + [H^+])/(10^{-8} + [H^+])$$

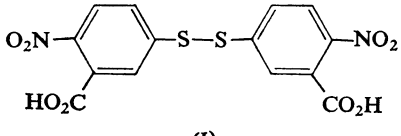
5. Papain reacts with both 2-Py–S–S–2-Py and 4-Py–S–S–4-Py in the pH range 2.5–8.5 to provide release of the thione fragments, stoicheiometric with the thiol content of the enzyme. 6. Whereas the ratios of the second-order rate constant for the reaction at pH4 to that at pH8 for the cysteine–2-Py–S–S–2-Py reaction ($k_{pH4}/k_{pH8} = 0.015$) and for the papain–4-Py–S–S–4-Py reaction ($k_{pH4}/k_{pH8} = 0.06$) are less than 1, that for the papain–2-Py–S–S–2-Py reaction is greater than 1 ($k_{pH4}/k_{pH8} = 15$). 7. This high reactivity of papain has been shown to involve reaction of the thiol group of cysteine-25, the enzyme's only cysteine residue, which is part of its catalytic site. 8. That this rapid and stoicheiometric reaction of the thiol group of native papain is not shown either by low-molecular-weight thiols or by the thiol group of papain after its active conformation has been destroyed by acid or heat denaturation, strongly commends 2-Py–S–S–2-Py as one of the most useful papain active-site titrants discovered to date. This reagent has been shown to allow accurate titration of papain active sites in the presence of up to 10-fold molar excess of L-cysteine and up to 100-fold molar excess of 2-mercaptoethanol.

Bender *et al.* (1966) have discussed the methods available for the determination of the absolute concentrations of active sites of hydrolytic enzymes and have convincingly demonstrated the preference for a stoicheiometric titration rather than for an assay based on measurement of rates of catalysis. These authors have specified the properties of the perfect titrant as follows: it should (1) be a specific substrate, (2) permit a titration to be made in only a few minutes for convenience and to obviate denaturation, (3) give a titration near neutral pH to reproduce physiological conditions, (4) be a stable, available and soluble reagent whose stoicheiometric reaction is easily detectable and (5) give a titration over a wide range of enzyme concentration. Exploitation of the 'burst' of *p*-nitrophenol ($k_{+2} \gg k_{+3}$) obtained on admixture of papain (EC 3.4.4.10) and certain *p*-nitrophenyl esters as the basis of an active-site titration is attractive, and Bender *et al.* (1966) selected *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester as the papain active-site titrant of choice. Unfortunately this substrate falls short of being a perfect titrant as defined above. Rapid deacylation of *N*-benzyloxycarbonyl-L-tyrosyl-papain and a low K_m value for the catalysis

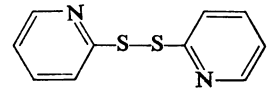
necessitates titration of the enzyme at pH3.2 and at papain concentrations of approx. 2μM. These conditions correspond to a 'burst' extinction change at 340nm of only approx. 0.01E unit. Further, the low water-solubility of this substrate necessitates the inclusion of organic solvent in the titration mixture.

Another type of titrant for thiol enzymes is a reagent that reacts stoicheiometrically with the enzyme to give a stable inactive derivative, i.e. an irreversible inhibitor. The use of an inhibitor rather than a substrate obviates the difficulties in the titration associated with the rapid turnover reaction. The chief disadvantage in using this type of reagent is that, at least near neutral pH, the reaction often appears to depend on uncomplicated chemical reaction of the active-site thiolate ion of the enzyme with the reagent and will not distinguish easily the thiol group in an intact active site from those in protein fragments, denatured enzyme or other thiol contaminants. This difficulty might be obviated by using an active-site-directed inhibitor (Baker, 1967), which hopefully would react much more rapidly with the thiolate ion of the enzyme than with contaminant thiols. Unfortunately, substrate analogues of papain that could inhibit the enzyme irreversibly in reactions that are easy to follow are not readily available. In spite of this, the nature of enzyme active sites leads one to expect the possibility of special effects even in reactions of group-specific reagents that are not formally substrate analogues. It may be envisaged that such effects can arise from interactions within the active site and/or between active site and reagent, leading to unusual reactivities as judged by reference to comparable reactions of the reagent with low-molecular-weight compounds. Such interactions may or may not be involved in the catalytic functioning of the enzyme. Cases in point are the unusually high reactivity of haloacetates with the active-site thiol group of papain (Sluyterman, 1968; Chaiken & Smith, 1969) and the saturation kinetics observed in the reactions of long-chain *N*-alkylmaleimides with this group (Anderson & Vasini, 1970). It seems likely that the probability of observing such special effects will be higher when the reagents contain ionizing substituents, which may serve as hydrogen-bonding sites. Three thiol reagents that are readily available and contain ionizing substituents are 5,5'-dithiobis-(2-nitrobenzoic acid) [Nbs_2, (I)], 2,2'-dipyridyl disulphide [2-Py–S–S–2-Py, (II)] and 4,4'-dipyridyl disulphide [4-Py–S–S–4-Py, (III)]. Neither 2-Py–S–S–2-Py nor 4-Py–S–S–4-Py has been investigated previously as an active-site titrant for papain. 2-Py–S–S–2-Py is of particular interest because of its apparent similarity in one respect to specific substrates for papain. This is shown in structure (IV). The requirement for an acylamino group in specific substrates for papain is well established (de Jersey, 1970), and this requirement is thought to involve binding of the amide moiety (Lucas & Williams, 1969). Model-building studies (Lowe & Yuthavong, 1971) have shown that the N–H linkage of the substrate's amide moiety could be bonded to the oxygen atom of the backbone carbonyl group of aspartic acid-158. In structure (IV) it is shown how a similar interaction could be involved in the binding of 2-Py–S–S–2-Py to papain.

Inspection of a molecular model of papain suggests that the interaction of 2-Py–S–S–2-PyH^+ with either the backbone carbonyl oxygen atom of aspartic acid-158 (IV) or with the carboxylate ion of the side



(I)



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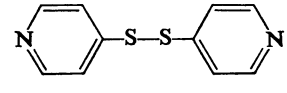

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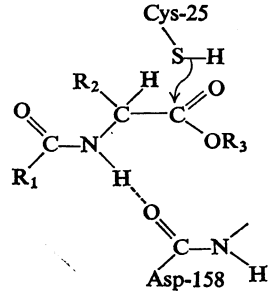

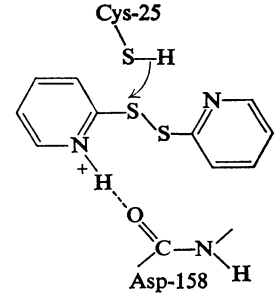

(IV)

chain of this residue, as we suggested previously (Brocklehurst & Little, 1970), could permit steric alignment of one or other of the sulphur atoms of the disulphide with the sulphur atom of cysteine-25. Nucleophilic attack at the sulphur atom further away from the positively charged nitrogen atom is the reaction that involves the better leaving group, i.e. the protonated incipient thiopyridone.

The titration of the papain thiol group by Nbs_2 has been reported by Robyt *et al.* (1971). These authors claimed to have shown that this reaction is complicated by reaction of Nbs^{2-} with the cystinyl disulphide bonds of papain, but we have since reported definitive evidence to show that this complication does not occur (Brocklehurst *et al.*, 1972).

The type of reaction in which the reagents (I)–(III) could react with the thiol group of cysteine-25 in papain is well documented (see Foss, 1961). This reaction, known as thiol–disulphide exchange or interchange, in the cases investigated kinetically, follows second-order kinetics (first-order in thiol and first-order in disulphide), and its mechanism is envisaged as nucleophilic attack of the thiolate ion on one of the sulphur atoms of the disulphide with subsequent or simultaneous expulsion of the fragment containing the other sulphur atom of the disulphide as a thiolate ion. The active-centre thiol group of papain has been implicated in such reactions. Sanner & Pihl (1963) interpreted the inhibition of papain by cystamine as formation of the mixed disulphide by thiol–disulphide exchange. Klein & Kirsch (1969) have shown that the inactive form of papain produced in the purification procedure of Kimmel & Smith (1954) is the mixed disulphide of the enzyme and L-cysteine. Sluyterman (1967) had demonstrated previously the identity of the rates of activation of inactive papain as prepared by the method of Kimmel & Smith (1954) and of the papain–cysteine mixed disulphide, and had shown that activation of the latter with low-molecular-weight thiols occurs by thiol–disulphide exchange.

Aromatic disulphides have been used to detect and estimate spectrophotometrically thiol groups in protein preparations. In such assays the thiol is allowed to react with an excess of the disulphide to produce a chromophoric fragment. The first study of this type was made by Barrnett & Seligman (1952), who used 2,2′-dihydroxy-6,6′-dinaphthyl disulphide to demonstrate histochemically protein-bound thiol groups. Ellman (1958) reported the use of bis-(*p*-nitrophenyl) disulphide for thiol determinations and later (Ellman, 1959) enhanced its water-solubility by introduction of carboxyl groups into the benzene rings. The extensive use of Ellman's (carboxylated) reagent [Nbs_2 (I)] in protein-modification studies has been reviewed by Glazer (1970). Grassetti & Murray (1967) reported the use of 2-Py–S–S–2-Py and 4-Py–S–S–4-Py as thiol titrants. Hitherto exploitation of the thiol–disulphide exchange reaction to investigate the active sites of thiol proteases has received little attention. There are examples of the use of Nbs_2 and similar reagents for determination of the thiol contents of proteolytic enzymes (e.g. see Wharton *et al.*, 1968). In such cases the extinction extrapolated to infinite time of the chromophoric fragment produced by the rapid reaction of the protein thiolate ion with the disulphide at pH approx. 8 is measured and used to compute the concentration of protein that possesses a reactive thiol group. This type of assay at this pH is unlikely to reveal any unusual reactivity that the protein thiol group might display as a result of its environment in the active site, and will probably not allow the distinction between the thiol groups in the active enzyme and other thiol groups that might be present.

We now report a spectrophotometric study of the reactions of papain with the disulphides (I)–(III) over a wide range of pH and an assessment of the compounds as active-site titrants for this enzyme. A prerequisite to this study was the determination of the spectrophotometric and ionization characteristics of the disulphides and of their products of interaction with thiols. A short account of some of this work has been presented previously (Brocklehurst & Little, 1970).

Materials and Methods

Papain

Three papain preparations were used and concordant results obtained with all of them: (i) the 2×-crystallized product of BDH Chemicals Ltd. (Poole, Dorset, U.K.); (ii) the 2×-crystallized product of Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); (iii) recrystallized papain prepared from BDH latex by the method of Kimmel & Smith (1954). The recrystallized enzyme was stored as a suspension in 50mM-sodium acetate buffer, pH4.5, at a concentration of approx. 20mg/ml.

Reagents

L-Cysteine hydrochloride and AnalaR EDTA were obtained from BDH Chemicals Ltd.; 2-mercaptoethanol was the 'pure' product of Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.); dithiothreitol was obtained from Calbiochem (Los Angeles, Calif., U.S.A.) and from Koch–Light Laboratories Ltd.; 2-thiopyridone (Py–2-SH) was the 'purum' product of Fluka A.-G. (Buchs SG, Switzerland); Nbs_2, 2-Py–S–S–2-Py and 4-Py–S–S–4-Py were obtained from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.); α-*N*-benzoyl-L-arginine ethyl ester was obtained from Koch–Light Laboratories Ltd.; *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester was obtained from BDH Chemicals Ltd.; Sephadex

G-25 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Preparation of stock solutions of the disulphides

Nbs_2. Only the anionic form of this reagent is sufficiently water-soluble to permit the preparation of aqueous stock solutions of useful concentration. Preparation of a solution of Nbs_2^{2-} in the absence of extraneous buffer was performed by slow addition of NaOH solution to a vigorously stirred suspension of Nbs_2. Local areas of strongly alkaline solution should be avoided because alkaline cleavage of the disulphide occurs. The neutralization was conveniently carried out in a pH-stat (Radiometer) at pH4 with 0.1M-NaOH in the syringe. Usually approx. 40mg of Nbs_2 was used to prepare 10ml of approx. 1mM stock solution. Undissolved disulphide was removed by filtration and the filtrate was stored at 4°C. Stock solutions of Nbs_2^{2-} were found to be highly susceptible to fungal spoilage, and solutions containing any trace of mycelium were discarded.

An effect that was observed but not further investigated was the reversible increase in colour intensity of Nbs_2^{2-} stock solutions when they were allowed to warm up to 25°C in the light from 4°C in the dark. Storage at 4°C in the dark overnight reversed the effect. Aromatic disulphides are known to exhibit thermochromism (Foss, 1961), and this phenomenon probably accounts for our observations. Nevertheless the precaution was taken of shielding stock solutions of Nbs_2^{2-} from the light with metal foil.

The concentration of Nbs_2^{2-} in the stock solution was determined as follows: 0.10ml of approx. 0.1mM-Nbs_2^{2-} solution was added to a large (at least 100-fold) molar excess of a low-molecular-weight thiol buffered at pH8 to give a total volume of 3ml in a spectrophotometric cell. The E_{412} value of the resulting solution was measured against a buffer–thiol blank and the concentration of Nbs_2^{2-} in the stock solution calculated by using Ellman's (1959) value of ϵ_{412} for Nbs^{2-} ($1.36\times10^4 M^{-1}\cdot cm^{-1}$) from $[Nbs_2^{2-}]_{stock} = 3\times10^{-3}E_{412}/2\times1.36$M. Several concentrations of thiol were used to check on the completeness of the reaction under these conditions. Progress-curve recording of E_{412} versus time established that under these conditions the exchange reaction occurs virtually instantaneously.

2-Py–S–S–2-Py and 4-Py–S–S–4-Py. Sufficiently concentrated aqueous stock solutions were prepared by dissolving the uncharged species in water, even though this species is relatively insoluble. About 40mg of the disulphide was added to 50ml of water at room temperature and the resulting suspensions were stirred magnetically for several hours. Insoluble material was then removed by filtration. The stock solutions of 4-Py–S–S–4-Py prepared in this way were approx. 3mM and those of 2-Py–S–S–2-Py approx. 1.5mM. These solutions were found to be stable for several months when stored at 4°C.

The concentrations of the disulphides were determined by scanning suitable dilutions of the stock solutions in potassium phosphate buffer, pH7, over the range 250–450nm against buffer blanks. Literature values (Grassetti & Murray, 1967) of the molar extinction coefficients of the neutral forms of 2-Py–S–S–2-Py and 4-Py–S–S–4-Py at their absorption maxima were used to calculate the Py–S–S–Py concentrations. Spectral scanning over the range of wavelength indicated permits an estimate of any thiopyridone contaminant.

Determination of the spectrophotometric and ionization characteristics of Nbs_2, 2-Py–S–S–2-Py, 4-Py–S–S–4-Py, Nbs, Py–2-SH and Py–4-SH

Spectrophotometry. The general method was to scan spectra of solutions of the disulphide or fragment at constant concentration in buffers (I0.1) at 25°C at intervals of 0.2 pH unit in the region of an ionization, and at wider pH intervals away from these regions. Spectra were recorded with either a Cary 15 or a Unicam SP.800 spectrophotometer. The pH of the contents of the spectrophotometer cell was measured immediately after each scan with Radiometer equipment.

The quantity $\bar{h}$ (see the Appendix) at a given pH was determined by using eqn. (1) for the compounds treated as monobasic acids (i.e. the thiol fragments) and eqn. (2) for the compounds treated as dibasic acids (i.e. the disulphides):

$$\bar{h} = \frac{E - E_{\mathrm{lim.\,(a)}}}{E_{\mathrm{lim.\,(b)}} - E_{\mathrm{lim.\,(a)}}} \quad (1)$$

$$\bar{h} = \frac{2[E - E_{\mathrm{lim.\,(a)}}]}{E_{\mathrm{lim.\,(b)}} - E_{\mathrm{lim.\,(a)}}} \quad (2)$$

In eqns. (1) and (2) E is the extinction at a given wavelength of a solution of the acid at a given concentration and pH, $E_{\mathrm{lim.\,(a)}}$ is the extinction at the same wavelength of a solution of the acid at the same concentration at a pH at which it is (essentially) totally in the acid form and $E_{\mathrm{lim.\,(b)}}$ the analogous extinction at a pH at which the compound is (essentially) totally in the base form. Eqn. (2) assumes that the difference molar extinction coefficients for the first and the second ionizations are the same. This is probably a reasonable assumption for disulphides, where there is little if any electronic transmission through the disulphide bond.

Potentiometric titration. Potentiometric titrations of the disulphides were attempted as follows. A stirred suspension of the neutral disulphide in aqueous KCl over which was passed a stream of O_2-free N_2 was titrated in a Radiometer autotitrator

with either 0.1M- or 1M-NaOH (CO_2-free) or HCl. Titrations were carried out several times both up and down the pH scale.

This method proved to be of limited usefulness. The limitation arose from the combination of the low pK_a values of the disulphides and the low solubilities of their neutral species. Useful results were obtained only for 4-Py-S-S-4-Py (see the Appendix).

Preparation of stock solutions of activated activator-free papain

Suspended papain (see above) was brought into solution by the addition of water with magnetic stirring at room temperature. To the resulting solution was added about 2½% of its volume of 0.1M-dithiothreitol solution. The pH of this solution was then raised to 8 by the addition of NaOH solution and the mixture was allowed to stand at room temperature for 15–30min. The activator was then removed on a Sephadex G-25 column. The eluent was deoxygenated water containing 10mM-KCl and 1mM-EDTA; O_2-free N_2 was bubbled through the eluent in the reservoir. Papain emerged from the column in the void volume, which was determined with Dextran Blue. The volume of solution applied to the column was normally 15–20ml and the bed volume of the column was approx. 75ml. Papain solution was collected in 5ml fractions and stored under O_2-free N_2 at 4°C. When only small batches of active enzyme were required, 3–4ml of solution was loaded on to a column of bed volume approx. 10ml.

Enzyme assays

Four types of assay were performed on papain during this investigation, namely determination of (1) the extinction at 280nm, (2) the amount of chromophoric fragment released on reaction of papain with Nbs_2, 2-Py-S-S-2-Py and 4-Py-S-S-4-Py, (3) the steady-state activity towards α-*N*-benzoyl-L-arginine ethyl ester and (4) the initial burst of *p*-nitrophenol released by reaction of papain with *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester.

Extinction at 280nm. This was measured on a papain solution prepared by dilution of the activated activator-free stock solution with water or pH7 buffer and the protein concentration was calculated by using $E^{1\%}_{1cm}$ 25 (Glazer & Smith, 1961) and molecular weight 23430 (Husain & Lowe, 1969).

Stoicheiometry of the reactions of Nbs_2, 2-Py-S-S-2-Py and 4-Py-S-S-4-Py with papain and the low-molecular-weight thiols, L-cysteine and 2-mercaptoethanol. The progress of these reactions was recorded spectrophotometrically by following the appearance of the chromophoric fragments Nbs^{2-} (at 412nm), Py-2-SH (at 343nm) and Py-4-SH (at 324nm) in a Cary 15, a Cary 16K or a Unicam SP.800 spectrophotometer. The solutions were preincubated in a thermostatically controlled bath at 25°C and water from the bath was circulated through the cell compartments of the spectrophotometer. A typical experiment is described. The extinction of a sample cell containing buffer (1.0ml to give final I0.1), 1mM-EDTA (2.0−x−$y$ml) and papain or thiol solution ($x$ml) was balanced against a reference cell containing buffer (1.0ml) and 1mM-EDTA (2.0−$y$ml) at the appropriate wavelength (see above) and a baseline was recorded. The reaction was started by rapid addition of disulphide solution ($y$ml) first to the reference cell and then to the sample cell. The progress curve was recorded until the reaction was complete and the pH of the contents of the sample cell was then measured. In the sample cell [thiol or papain] was usually 0.005–0.1mM and [disulphide] was usually 0.01–0.5mM. In all experiments disulphide concentration was greater than the thiol or papain concentration. The final concentration of the fragment released from the disulphide was computed from its extinction coefficient at the pH of the experiment. For reactions in which the papain thiol group reacted stoicheiometrically with the disulphide, as deduced from the constancy of the extinction change obtained with several different concentrations of the disulphide, the thiol content of the papain concentration was calculated by dividing the concentration obtained in this assay by that obtained from measurement of E_{280}.

Steady-state activity of papain towards α-N-benzoyl-L-arginine ethyl ester. This was measured with a Radiometer pH-stat. The titrant was usually 0.1M-NaOH and the titrant reservoir was protected from atmospheric CO_2 by self-indicating Carbosorb. Solutions were preincubated at 25°C and the reaction was followed in a jacketed cell thermostatically controlled with water from the preincubation bath. Water-saturated O_2-free N_2 was passed over the cell contents during the assay. A reaction cell was prepared typically by mixing 0.5M-KCl (1.0ml), 0.1M-EDTA (0.5ml), α-*N*-benzoyl-L-arginine ethyl ester solution ($x$ml) and water (3.95−x−$y$ml). This mixture was adjusted to the required pH by addition of a small, negligible, volume of concentrated NaOH or constant-boiling HCl and the reaction was started by addition of enzyme solution ($y$ml). The enzyme concentration was usually approx. 5μM and the substrate concentration was usually 20mM in the cell.

In view of the curved traces obtained in the absence of extraneous thiol, rates were obtained from slopes of equivalent early portions of the progress curves. When inclusion of extraneous thiol was permissible this was L-cysteine, 2-mercaptoethanol or dithiothreitol. In these cases the enzyme and thiol were allowed to equilibrate in the reaction cell and the reaction was started by addition of substrate.

When a thiol was included in the reaction mixture, linear progress curves were obtained.

Active-site titration of papain by N-benzoyloxycarbonyl-L-tyrosine p-nitrophenyl ester. The method used was essentially that of Bender *et al.* (1966).

Effect of 2-Py–S–S–2-Py on the catalytic activity of papain towards α-N-benzoyl-L-arginine ethyl ester at pH3.8

The catalytic activity of a sample of papain towards this substrate was determined in a pH-stat as described above. A solution of the substrate containing substrate (0.20ml of 0.5M stock solution), EDTA (0.05ml of 0.1M solution), KCl (1.0ml of 2M solution) and water (3.7ml) was equilibrated at pH3.8. The reaction was started by addition of 0.05ml of a solution of activated activator-free papain (0.34mM-SH group; 0.46mol of SH group/mol of protein). This gives a concentration in the cell of 3.4μM for enzyme displaying a reactive thiol group. The progress curve of the reaction was recorded for a few minutes and then a solution of 2-Py–S–S–2-Py (0.10ml of 1.5mM solution) was added to give a concentration in the cell of 0.03mM for 2-Py–S–S–2-Py and recording was continued.

Preparation of papain blocked by reaction with 2-Py–S–S–2-Py at pH3.8, free from excess of 2-Py–S–S–2-Py (and control)

To two identical solutions each composed of 1.0ml of a solution of activated activator-free papain (0.34mM-SH group; 0.46mol of SH group/mol of protein) buffered with 0.25ml of sodium acetate buffer, pH3.8 and *I*0.3, were added in one case 1.0ml of 1.5mM-2-Py–S–S–2-Py and in the other 1.0ml of water. The pH of each solution was checked and they were then kept at 4°C. After 10min the solutions were passed (separately) through a Sephadex G-25 column (bed volume approx. 10ml) with aqueous eluent containing 10mM-KCl and 1mM-EDTA. A 2.5ml volume of the eluate was collected in each case and stored under N_2 at 4°C.

Assays performed on 2-Py–S–S–2-Py-blocked papain and on papain kept at pH3.8 as a control

Protein concentration. This was determined by measurement of E_{280}.

Concentration of papain-bound Py–2-SH groups. This was determined spectrophotometrically after releasing Py–2-SH by reaction of the mixed disulphide with 2-mercaptoethanol: 0.15ml of the solution obtained from the Sephadex column was added to 2.8ml of Tris–HCl buffer, pH7.6 or 8.0 and *I*0.1, in a spectrophotometer cell. The extinction of this solution was recorded over the range 300–450nm against a buffer blank. 2-Mercaptoethanol (0.01ml) was then added to both reference and sample cells (giving a final concentration of approx. 50mM) and the spectrum was recorded as before. Repeated scanning of the reaction mixture showed that under these conditions release of Py–2-SH is virtually instantaneous. The concentration of Py–2-SH released was calculated by using $\epsilon_{343} = 7.06\times10^3 M^{-1}\cdot cm^{-1}$.

Thiol content. This was determined by reaction with Nbs_2^{2-} at pH8 and with 2-Py–S–S–2-Py at pH7.6 (see above).

Activity towards α-N-benzoyl-L-arginine ethyl ester. This was determined as described above. A solution of the substrate containing substrate (0.10ml of 0.5M solution), EDTA (0.05ml of 0.1M solution), KCl (1.0ml of 2M solution) and water (3.7ml) was equilibrated in a pH-stat at pH6.5. The reaction was started by addition of 0.15ml of the solution from the Sephadex G-25 column.

The activity was determined in the absence and in the presence of extraneous thiol by the addition of 0.01ml of 2-mercaptoethanol to the reaction mixture during the assay to give a concentration of approx. 30mM.

Change in catalytic activity towards α-N-benzoyl-L-arginine ethyl ester at pH6.5 and in reactivity towards 2-Py–S–S–2-Py at pH3.8 during the acid denaturation of papain

To 1ml of a solution of activated activator-free papain (0.255mM-SH group) was added 0.25ml of glycine–HCl buffer, pH2.6 and *I*0.3. The mixture was kept at 25°C, and samples were removed at intervals and assayed for activity towards α-*N*-benzoyl-L-arginine ethyl ester at pH6.5 (0.05ml portions) and for reaction with 2-Py–S–S–2-Py at pH3.8 (0.10ml or 0.20ml portions).

Results and Discussion

Spectrophotometric and ionization characteristics of Nbs_2, 2-Py–S–S–2-Py, 4-Py–S–S–4-Py, Nbs, Py–2-SH and Py–4-SH

Before a study of the reactions of the papain thiol group with Nbs_2, 2-Py–S–S–2-Py and 4-Py–S–S–4-Py was made it was necessary to know how the absorption spectra of the products of these thiol–disulphide exchanges, i.e. Nbs, Py–2-SH and Py–4-SH, vary with pH in order to calculate their concentrations at a given pH. It was desirable to know also the ionization characteristics of these compounds and those of the disulphides from which they are derived to ascertain where pH-dependent changes in the rates and equilibrium positions of the thiol–disulphide exchanges might be expected to occur.

The u.v.-spectral and ionization characteristics of these compounds are collected in Table 1 of the Appendix.

Reactions of papain, L-cysteine and 2-mercaptoethanol with Nbs_2, 2-Py–S–S–2-Py and 4-Py–S–S–4-Py

The progress of these reactions was followed spectrophotometrically by recording the formation of Nbs from Nbs_2, Py-2-SH from 2-Py–S–S–2-Py and Py–4-SH from 4-Py–S–S–4-Py in the pH range 2.5–8.5. A detailed kinetic analysis of some of these reactions has been reported (Brocklehurst & Little, 1972; Little & Brocklehurst, 1972). The pH range over which these reactions could be studied conveniently by using conventional spectrophotometric techniques was limited when one of the following conditions obtained: (1) protonation or deprotonation of the thiol fragment released in the interchange resulted in a decrease of its extinction coefficient at its $\lambda_{max.}$ to a degree that prevented accurate progress-curve recording; (2) OH^- ion cleavage of disulphide became kinetically significant; (3) papain became denatured at low pH (below approx. pH3); (4) reactions became so fast that the progressive decrease in the thiol concentration (to permit use of lower disulphide concentrations) reached the point at which ΔE became inadequate for accurate determination of rate constants.

Reactions of the low-molecular-weight thiols with Nbs_2, 2-Py–S–S–2-Py and 4-Py–S–S–4-Py

Under the conditions of concentration used in this study (see the Materials and Methods section) the reactions of both low-molecular-weight thiols with all three disulphides (in excess) resulted in the stoicheiometric release of Nbs, Py–2-SH or Py–4-SH. The rates of these reactions are considerably faster at pH values approx. 8 than at pH values approx. 4. This suggests that the predominant reaction pathway in approximately neutral media is nucleophilic attack of the thiolate ion on the unprotonated disulphide. This conclusion is supported for at least some of these reactions by the kinetic analysis reported previously (Brocklehurst & Little, 1972; Little & Brocklehurst, 1972).

Reaction of papain with Nbs_2

The extinction changes in the reactions of Nbs_2 with L-cysteine and with 2-mercaptoethanol in the pH range 4–8 were those predicted from the pH-dependence of the extinction coefficient of Nbs^{2-} and the concentrations of the thiols in the reaction mixtures, assuming that stoicheiometric reactions had occurred. By contrast, the corresponding extinction changes for the reaction of papain and Nbs_2 at pH values less than 7 were less than those calculated for a stoicheiometric reaction. Measurement of the extinction changes at constant pH, constant papain concentration and various Nbs_2 concentrations confirmed that the small extinction changes for the reactions in the acid region can be accounted for by a decrease in the equilibrium constant ($K_{equil.}$) with decrease in pH. Variation of $K_{equil.}$ with pH is shown in Fig. 1 and a plot of $pK_{equil.}$ versus pH is shown in Fig. 2. The latter may be interpreted by using Dixon's rules (Dixon, 1953). In Fig. 2 the following features are apparent: (i) a line of unit negative slope in the pH region 5.5–7.5, which suggests that in this region the product state is of charge −1 relative to the reactant state; (ii) the possibility that the experimental values of $pK_{equil.}$ might lie above the straight line mentioned in (i) at pH values above 7.5; if true this would suggest a change to zero slope in the alkaline region, i.e. a $pK_a > 7.5$ unique to the reactant state; (iii) a tendency for the experimental values of $pK_{equil.}$ to lie below the line at pH values below 5.5, which suggests a change to zero slope in the acid region, i.e. a $pK_a < 5.5$ unique to the product state.

Attempted determinations of $K_{equil.}$ at pH values below 5 and above 8 were subject to very considerable error. Protonation of Nbs^{2-} (pK_a 4.41) with resulting fall in extinction coefficient in the low-pH region and the approach of the extinction change accompanying the reaction to that for a stoicheiometric reaction in the high-pH region made determinations of $K_{equil.}$ in these regions unreliable.

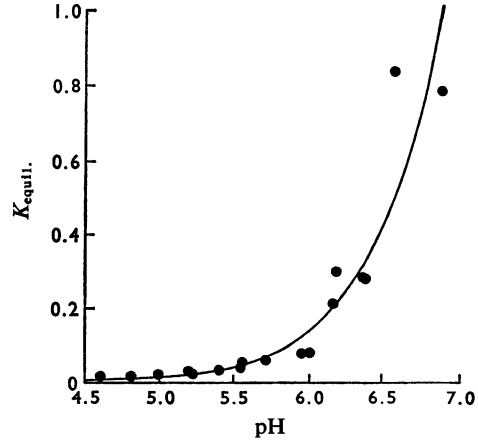

Fig. 1. *pH-dependence of $K_{equil.}$ for the reversible reaction of papain with Nbs_2*

The reaction was carried out at 25.0°C and I 0.1. The line is theoretical for $K_{equil.} = 3.5 \times 10^{-3}$ $(3.92 \times 10^{-5} + [H^+])/(10^{-8} + [H^+])$ (see the text).

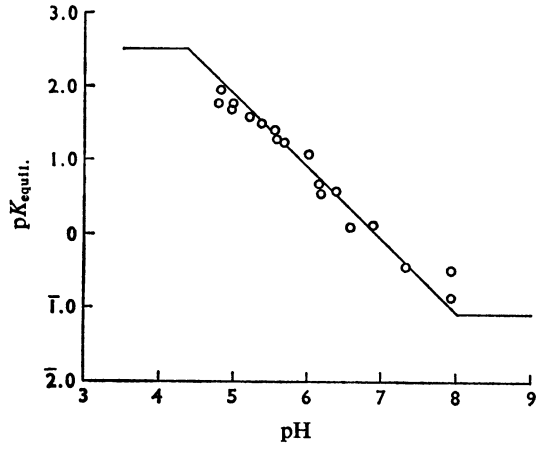

Fig. 2. *pH-dependence of* $pK_{equil.}$ *for the reversible reaction of papain with* Nbs_2

The reaction was carried out at 25.0°C and I0.1. The line of slope −1 is shown intersecting lines of zero slope at pH values 4.41 and 8.0, which are the pK_a values of the thiol groups of Nbs and papain respectively (see the text).

Greatest confidence can be placed in the $K_{equil.}$ values in the pH region 5–7.

It seems reasonable to identify the pK_a<5.5 suggested by Fig. 2 as pK_{a_2} of Nbs, which describes the ionization of its thiol group, and the possible pK_a>7.5 as the pK_a of the papain thiol group. The $pK_{a_{II}}$ of Nbs determined spectrophotometrically is 4.41 (see Table 1 of the Appendix) and the pK_a of the papain thiol group determined kinetically in its reaction with Nbs_2^{2-} is 8.0 (Little & Brocklehurst, 1972). The simplest description of this equilibrium system is thus given by:

$$K_{equil.} = 3.5 \times 10^{-3} (3.92 \times 10^{-5} + [H^+])/(10^{-8} + [H^+])$$

The value of the pH-independent equilibrium constant, $\bar{K}_{equil.}$ (3.5×10^{-3}), is obtained by extrapolation of the line of slope −1 in Fig. 2 to pH4.41.

Reaction of papain with 4-Py–S–S–4-Py

Papain reacts with 4-Py–S–S–4-Py in the pH range 2.5–8.5 to provide release of Py–4-SH that is stoicheiometric with the thiol content of papain (usually 0.4–0.6mol of SH group/mol) as assessed by titration with Nbs_2^{2-} at pH8.0 and with *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (see the Materials and Methods section). A detailed kinetic study of this reaction has been presented (Brocklehurst & Little, 1972). It is sufficient for the purposes of the present paper to report that, as with the reactions of 4-Py–S–S–4-Py with low-molecular-weight thiols, the reactions of 4-Py–S–S–4-Py with papain are slow at pH values approx. 4 and fast at pH values approx. 8.

Reaction of papain with 2-Py–S–S–2-Py

The reaction of papain with 2-Py–S–S–2-Py in the pH range 2.5–9.5 provides release of Py–2-SH that is stoicheiometric with the thiol content of papain. In this respect the reaction resembles the reaction of papain with 4-Py–S–S–4-Py. In marked contrast with this latter reaction and the reactions of low-molecular-weight thiols with 2-Py–S–S–2-Py, however, the reaction of papain with 2-Py–S–S–2-Py is very much faster at pH values 3.5–4.5 than at pH values 6–9.5. For example, the ratios of the second-order rate constant for reaction at pH4 to that at pH8 for these three systems are: L-cysteine–2-Py–S–S–2-Py, $k_{pH4}/k_{pH8} = 0.015$; papain–4-Py–S–S–4-Py, $k_{pH4}/k_{pH8} = 0.06$; papain–2-Py–S–S–2-Py, $k_{pH4}/k_{pH8} = 15$. Thus this ratio of reactivities is approx. 10^3 times greater in the reaction of papain with 2-Py–S–S–2-Py than in the reaction of L-cysteine with 2-Py–S–S–2-Py. Similar results were obtained with papain from the three sources given in the Materials and Methods section.

Nbs_2, 2-Py–S–S–2-Py and 4-Py–S–S–4-Py as active-site titrants for papain

The low equilibrium constant for the reaction of papain with Nbs_2 and its pH-dependence in the acid region make Nbs_2 unsuitable for use as a papain titrant except possibly at pH8 and above. Even at pH8 Nbs_2 reacts at comparable rates with both papain and low-molecular-weight thiols. Thus the objection to the use of most inhibitors as active-site titrants, i.e. their inability to distinguish functional groups in intact enzyme active sites from the same functional groups in other molecules (see the introduction), applies to this reagent. This objection applies equally to 4-Py–S–S–4-Py, which could be considered a better titrant than Nbs_2 for papain because the equilibrium constant for this reaction is high and essentially pH-independent, at least below pH9. The advantages of 4-Py–S–S–4-Py and 2-Py–S–S–2-Py as thiol titrants that result from their essentially irreversible reactions with thiols have been reported by Grassetti & Murray (1967).

The high reactivity of papain towards 2-Py–S–S–2-Py at low pH, which is not exhibited by the low-molecular-weight thiols L-cysteine and 2-mercaptoethanol, suggested that this reagent might conform to the requirements for a perfect titrant for papain (see the introduction). Accordingly, further experiments were carried out to establish beyond reasonable doubt that this reaction occurs at the thiol group of intact papain active sites. That essentially the same

stoicheiometry, that corresponding to the enzyme thiol as determined by other methods, is observed at all pH values in the reaction of papain with 2-Py–S–S–2-Py suggests that the same group, probably the enzyme's only thiol group, which is in the side chain of the active-site cysteine residue (residue 25) (Light *et al.*, 1964; Drenth *et al.*, 1968), is the reaction site for 2-Py–S–S–2-Py at all pH values. Although what is presumably a nucleophilic attack on 2-Py–S–S–2-Py that is much faster at pH4 than at pH8 is completely uncharacteristic of a thiol group, the view that this is indeed the reactive centre in the enzyme is compelled by the stoicheiometry of the reactions at different pH values together with the following evidence. Papain that had been allowed to react with a 10-fold molar excess of 2-Py–S–S–2-Py at pH3.8 and separated from the excess of 2-Py–S–S–2-Py on a Sephadex G-25 column failed to react either with 2-Py–S–S–2-Py at pH7.6 or with Nbs_2^{2-} at pH8.0. A control sample of enzyme that had been allowed to stand at pH3.8 for an equivalent period of time followed by passage through a Sephadex G-25 column was found to contain the expected thiol content as determined by titration with 2-Py–S–S–2-Py and with Nbs_2^{2-}. Papain blocked with 2-Py–S–S–2-Py at pH3.8 and separated from the excess of reagent was completely inactive towards α-*N*-benzoyl-L-arginine ethyl ester at pH6.5. Treatment of the 2-Py–S–S–2-Py-blocked papain with an excess of 2-mercaptoethanol at pH8.0 both released the expected quantity of Py–2-SH and regenerated the expected activity of the enzyme towards α-*N*-benzoyl-L-arginine ethyl ester.

Evidence has been presented previously (Brocklehurst & Little, 1972) that the high reactivity of the papain thiol group towards 2-Py–S–S–2-Py at pH values approx. 4 results from reaction of the sulphur atom of the cysteine-25–histidine-159–asparagine-175 hydrogen-bonded system with the reagent protonated on the nitrogen atom.

Changes in catalytic activity towards α-N-benzoyl-L-arginine ethyl ester at pH6.5 and reactivity towards 2-Py–S–S–2-Py at pH3.8 consequent on the acid denaturation of papain

The experiments described above demonstrate that at pH3.8 the rapid reaction of 2-Py–S–S–2-Py with papain is a thiol–disulphide interchange involving the enzyme's only thiol group. To ascertain whether there is any relationship between the conformation of the papain molecule that is required for catalytic activity and the conformation that may be required for the unusually high reactivity of papain with 2-Py–S–S–2-Py at low pH, we investigated the changes in both of these types of activity during acid denaturation of papain at pH2.6 and 25°C. After approx. 1h the denaturation process, as measured by the fall in catalytic activity of the enzyme towards α-*N*-benzoyl-L-arginine ethyl ester at pH6.5, appeared to reach an equilibrium position in which about 60% of the active papain originally present was inactive. Although reaction of papain with 2-Py–S–S–2-Py at pH3.8 at the beginning of the slow denaturation process was virtually instantaneous, which is characteristic of the rate of reaction of active papain with 2-Py–S–S–2-Py at the concentration employed (0.05mM), the reaction of the equilibrium mixture of active and inactive enzyme with 2-Py–S–S–2-Py was clearly biphasic. A virtually instantaneous reaction was followed by a much slower reaction, which reached an extinction reading (for the total reaction) corresponding to the total thiol content of the mixture of inactive and active enzyme. Typical progress curves for the reaction of active papain and the equilibrium mixture of active and acid-denatured papains are shown in Fig. 3, and the progress curve for the acid-denaturation process together with stoicheiometries of the rapid 2-Py–S–S–2-Py reactions are shown in Fig. 4. The initial portion of the progress curve of the slow phase of the reaction (Fig. 3) was extrapolated to zero time to give the stoicheiometry of the initial fast phase. The correspondence between the fall in catalytic activity towards α-*N*-benzoyl-L-arginine ethyl ester and the fall in the stoicheiometry of the initial fast reaction with 2-Py–S–S–2-Py demonstrates that the thiol group of native papain shows a markedly higher reactivity towards 2-Py–S–S–2-Py at pH3.8 than does the thiol group of the acid-denatured enzyme.

Preliminary experiments, in which higher temperature was used to effect the denaturation of papain rather than low pH, have indicated similar parallel changes in catalytic activity and stoicheiometry of the fast reaction with 2-Py–S–S–2-Py at pH3.8. In some of the papain preparations used the fast reaction of the enzyme with 2-Py–S–S–2-Py at pH3.8 was followed by a much slower reaction. The extent of the slow phase was about 5–10% of the total extinction change and was taken to represent reaction of small amounts of denatured enzyme. With such papain preparations the thiol content of the enzyme as determined by the instantaneous phase of the reaction with 2-Py–S–S–2-Py at pH3.8 was 5–10% lower than that measured by reaction with 2-Py–S–S–2-Py at pH7.

Titration of intact papain active sites in the presence of low-molecular-weight thiols

The much faster reaction of 2-Py–S–S–2-Py at pH3.8 with papain than with either L-cysteine or 2-mercaptoethanol suggested that it should be possible to determine the concentration of active papain even in the presence of these thiols. By extrapolation of the initial portion of the slow second phase of the reaction (reaction of 2-Py–S–S–

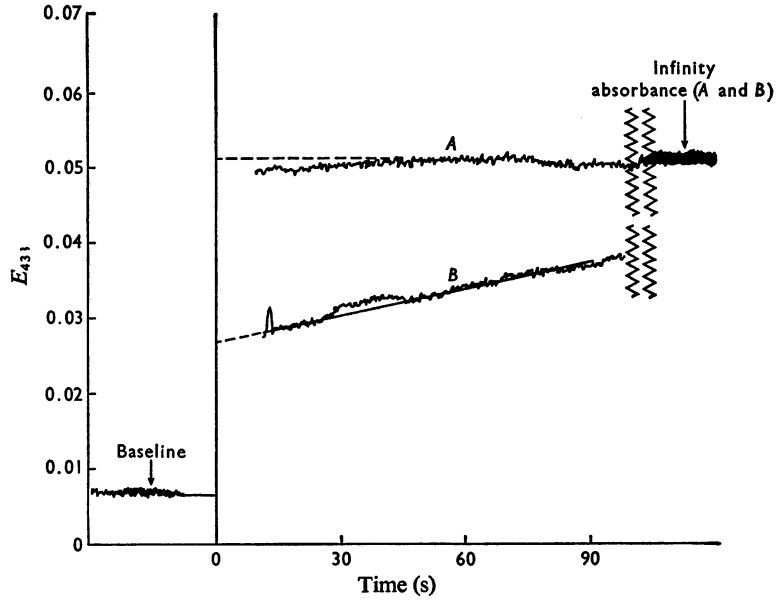

Fig. 3. *Typical progress curves for the reactions of* 2-*Py–S–S–*2-*Py at pH*3.8 *with active papain and with the mixture of active and inactive papain resulting from acid denaturation*

Curve *A*, reaction with active papain; curve *B*, reaction with the mixture of active and inactive papain resulting from acid denaturation (see the text and Fig. 4). The reactions were carried out at 25.0°C and *I*0.1. The concentration of papain was 8.5 μM (protein) and that of 2-Py–S–S–2-Py was 0.05 mM.

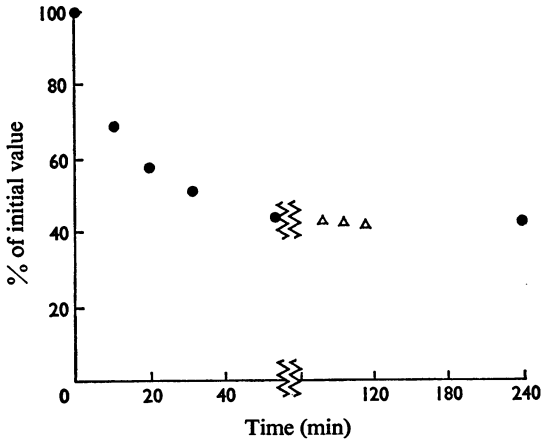

Fig. 4. *Effects of acid denaturation of papain at pH*2.6, 25.0°*C and I*0.1 *on its catalytic activity towards α-N-benzoyl-*L*-arginine ethyl ester at pH*6.5 *and the stoicheiometry of the fast phase of the reaction with* 2-*Py–S–S–*2-*Py at pH*3.8

●, Catalytic activity towards α-*N*-benzoyl-L-arginine ethyl ester; △, stoicheiometry of the fast phase of the reaction with 2-Py–S–S–2-Py. For details see the text.

2-Py with the low-molecular-weight thiol) to zero time it was possible to titrate active papain in the presence of L-cysteine at up to 10 times molar excess of the latter and in the presence of mercaptoethanol, which has a higher pK_a value than L-cysteine, at up to 100 times molar excess.

Assessment of 2-*Py–S–S–*2-*Py as a papain active-site titrant*

It is clear that the rapidity of the reaction of activated papain with 2-Py–S–S–2-Py at pH3.8 is a function of the particular geometry that characterizes the papain active site. Such high reactivity towards 2-Py–S–S–2-Py is not shown by low-molecular-weight thiols, nor by the thiol group of papain whose conformation has been altered either by acid or heat denaturation. It would seem that the high reactivity of the thiol group of native papain towards 2-Py–S–S–2-Py is a function also of the geometry of the 2-Py–S–S–2-Py molecule, whether reflected in its binding ability or in its electrophilic character, since similar high reactivity at low pH is not observed in the reaction of papain with 4-Py–S–S–4-Py.

Titration with 2-Py–S–S–2-Py may be carried out as rapidly as with *N*-benzyloxycarbonyl-L-tyrosine

p-nitrophenyl ester; it is performed at a somewhat higher pH, where denaturation is less rapid, and, since there is no turnover in the reaction, plots of $1/\sqrt{\pi}$ versus $1/[S_0]$ (see Bender *et al.*, 1966) are not required. 2-Py-S-S-2-Py is stable for at least several months when stored as a saturated aqueous solution at 4°C. Its water-solubility (1.5 mM) is sufficient for the purposes here described and obviates the necessity for inclusion of organic solvent in the reaction mixture. Although the values of $\Delta\epsilon$ for the two titrations are about the same [6.2×10^3 M$^{-1}\cdot$cm^{-1} for *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (Bender *et al.*, 1966) and 7.1×10^3 M$^{-1}\cdot$cm^{-1} for 2-Py-S-S-2-Py], the 2-Py-S-S-2-Py titration may be used over a wide range of enzyme concentration whereas the former titration is severely limited in this respect.

While this work was in progress, Williams & Lucas (1970) reported the synthesis of α-*N*-benzyloxycarbonyl-D-norleucine *p*-nitrophenyl ester and its use as a papain titrant. Although this reagent inevitably shows some of the drawbacks associated with 'burst' titrants (see the introduction), it does allow titration of the enzyme in approximately neutral media. This is possible because its D-configuration results in the formation of an acyl-papain whose deacylation is characterized by a low rate constant.

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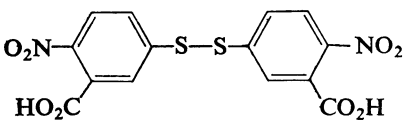
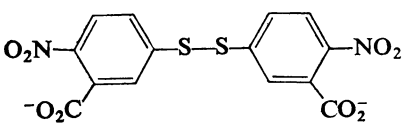
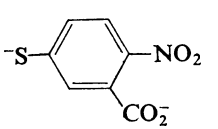
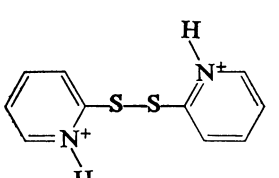
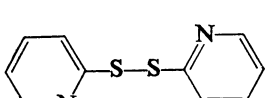
APPENDIX

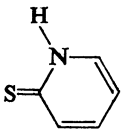
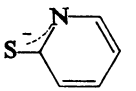
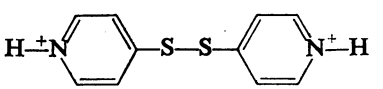
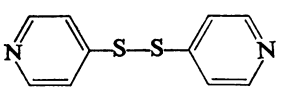
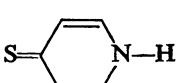
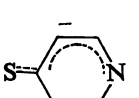
Spectrophotometric and ionization characteristics of Nbs$_2$, 2-Py-S-S-2-Py, 4-Py-S-S-4-Py, Nbs, Py-2-SH and Py-4-SH

These are summarized in Table 1.

The three aromatic disulphides behave as dibasic acids in which the ionizing groups are carboxyls in Nbs$_2$ and pyridinium ions in 2-Py-S-S-2-Py and in 4-Py-S-S-4-Py. The ionizations may be described both in terms of intrinsic group dissociation constants (K_{a_1}–K_{a_4}) and in terms of molecular acid dissociation constants (K_{a_I} and $K_{a_{II}}$). These constants are defined and their inter-relationships discussed by Edsall & Wyman (1958). Since all the disulphides are symmetrical, $K_{a_1} = K_{a_3}$ and $K_{a_2} = K_{a_4}$. Strictly Nbs, Py$^+$H-2-SH and Py$^+$H-4-SH also are dibasic acids. In these cases, however, the acid groups (thiol on the one hand and carboxyl and pyridinium on the other) have very different intrinsic dissociation constants (see below) and the two successive stages of ionization may, for practical purposes, be dealt with

Table 1. *U.v.-spectral and ionization characteristics of Nbs$_2$, 2-Py–S–S–2-Py, 4-Py–S–S–4-Py, Nbs, Py-2-SH and Py–4-SH at 25.0°C and I 0.1*

Compound	Molecular dissociation constants pK_{a_I}	Molecular dissociation constants p$K_{a_{II}}$	Group dissociation constants pK_{a_1}	Group dissociation constants pK_{a_2}	$\lambda_{max.}$ (nm)	$10^4 \epsilon_{max.}$ ($M^{-1} \cdot cm^{-1}$)	Reference
Nbs_2	1.57*	2.15*	1.87*	1.85*			Present work
					325		Present work
					325	1.8	Present work
Nbs	—	4.41	—	~4.4			Present work
					412	1.36	Ellman (1959)
2-Py–S–S–2-Py	<1	2.45	—	2.15			Present work
					300	≥1.34†	Present work
					281	0.97	Grassetti & Murray (1967)

Py-2-SH		9.80				Present work
	−1.07	9.97				Albert (1963)
				271	1.04	Grassetti & Murray (1967)
				343	0.706	Grassetti & Murray (1967)
				263	1.1	Present work
				310	0.43	Present work
4-Py-S-S-4-Py	4.00	5.11	4.30 4.81			
				280	2.16	Present work
				247	1.63	Grassetti & Murray (1967)
Py-4-SH	1.43	8.83	— —			Albert (1963)
		8.60				Present work
				230	0.96	Grassetti & Murray (1967)
				324	1.98	Grassetti & Murray (1967)
				284		Present work

* Assuming both of the Nbs_2 carboxyl groups are titrated (see the text).
† From a scan in conc. HCl.

separately as though each were that of a monobasic acid. For these compounds K_{a_I} will approximate to either K_{a_1} or K_{a_3} and $K_{a_{II}}$ will approximate to either K_{a_2} or K_{a_4}. Also by contrast with the situation that obtains in the disulphides there is reason to suppose that $K_{a_1} \neq K_{a_4}$ and $K_{a_2} \neq K_{a_3}$ (see below).

It is convenient to define $\bar{h}$ as the average number of protons lost per molecule from the fully protonated form of an acid (see Edsall & Wyman, 1958). For a monobasic acid whose ionization is described by eqn. (1), $\bar{h}$, defined by eqn. (2), varies between 0 and 1:

$$AH^{n+} \underset{}{\overset{K_a}{\rightleftharpoons}} A^{(n-1)+} + H^+ \qquad (1)$$

$$\bar{h} = \frac{[A^{(n-1)+}]}{[AH^{n+}] + [A^{(n-1)+}]} = \frac{1}{1 + [H^+]/K_a} \qquad (2)$$

For a dibasic acid whose ionization is described by eqn. (3), $\bar{h}$, defined by eqn. (4), varies between 0 and 2:

$$AH_2^{n+} \overset{K_{a_I}}{\rightleftharpoons} AH^{(n-1)+} + H^+ \rightleftharpoons A^{(n-2)+} + H^+ \qquad (3)$$

$$\bar{h} = \frac{[AH^{(n-1)+}] + 2[A^{(n-2)+}]}{[AH_2^{n+}] + [AH^{(n-1)+}] + [A^{(n-2)+}]} \qquad (4)$$

$$= \frac{1 + 2K_{a_{II}}/[H^+]}{1 + [H^+]/K_{a_I} + K_{a_{II}}/[H^+]}$$

To determine molecular acid dissociation constants from pH–$\bar{h}$ data it is convenient to use linear transforms of eqns. (2) and (4), i.e. the reciprocal form of eqn. (2) and eqn. (5):

$$\bar{h}[H^+]/(\bar{h}-1) = \{(2-\bar{h})\,K_{a_1}K_{a_{II}}/(\bar{h}-1)[H^+]\} - K_{a_I} \qquad (5)$$

Nbs_2. The decrease in extinction at the $\lambda_{max.}$ of the dianion (325nm) that occurs consequent on protonation follows a titration curve that could represent a single protonation. The insolubility of Nbs_2 prevented the potentiometric titration required to resolve the common ambiguity of spectrophotometric titrations that obtains in this case. This arises from not knowing the extinction coefficients of particular ionic forms of a compound. Thus the titration of Nbs_2 may represent equally well the dissociation of both of the carboxyl groups of Nbs_2 if there is no interaction between them with $pK_{a_I} = 1.57$ and $pK_{a_{II}} = 2.15$ or the dissociation of Nbs^{2-} with $pK_{a_{II}} = 1.82$. In the latter case, pK_{a_I} of Nbs_2 must be less than 1.

2-Py–S–S–2-Py. A large spectral change accompanies protonation of 2-Py–S–S–2-Py; the absorption band at 280nm shifts to 300nm. Analysis of the pH–E_{300} data in the pH range 1–4 provides evidence of a single protonation. That these data do not represent the ionization of two identical non-interacting groups, as may be the case with Nbs_2, is suggested by the large spectral changes (increase in E_{300} and shift in isosbestic point) observed below pH1. Repeated spectral scanning in 10M-HCl confirmed that destruction of 2-Py–S–S–2-Py was not proceeding at a significant rate.

4-Py–S–S–4-Py. Protonation of 4-Py–S–S–4-Py ($\lambda_{max.}$ 247nm) results in a shift of the absorption band to 280nm; a shifting isosbestic point was observed. The relatively high pK_a values of this compound allowed the recording of its potentiometric titration curve (25°C, I 0.1, [4-Py–S–S–4-Py] = 25mM, 1M titrant) without solvent titration necessitating major corrections. Even in this case, the neutral species, which has a solubility in water at 25°C of approx. 3mM, did not remain in solution. It was possible, however, to confirm that titration in the pH range 2–7 required two equivalents of acid or alkali.

Nbs. $K_{a_{II}}$, which will approximate K_a for the thiol group of Nbs^-, was determined by measuring E_{412} in the pH range 3–8. A stock solution of Nbs^{2-} was prepared by mixing Nbs_2^{2-} stock solution with a large excess of 2-mercaptoethanol in water or dilute buffer at pH7. Incorporation of further quantities of 2-mercaptoethanol into the reaction mixtures without further change in E_{412} confirmed that quantitative conversion of Nbs_2^{2-} into Nbs^{2-} had been achieved. The resulting pK_a (4.41) is identical with that reported by Airee (1967) and closely similar to that for *p*-nitrothiophenol (4.39±0.1) reported by Wharton (1969).

Py–2-SH and Py–4-SH. The protonic equilibria that exist in aqueous solutions of these compounds have been reported by Albert (1963). In the present study, values of $pK_{a_{II}}$ were determined for Py–2-SH by analysis at 343nm and for Py–4-SH by analysis at 324nm.

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